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[54]发明名称 抗肿瘤抗生素力达霉素的制备新方法

[57]摘要

本发明涉及一种大分子肽类抗肿瘤抗生素力达霉素的制造新方法，由于力达霉素分子中肽与发色团以非共价键结合，外界条件如有机溶媒、pH、洗脱剂离子特性、光和热等因素影响其结合并易引起发色团失活，采用羟基磷灰石柱吸附工艺，缩短流程，避光操作，可产生不同于一般离子交换层析的特殊分离效果，既提高了制品纯度，也增强了它的稳定性，生物活性实验证明，采用精原细胞法和克隆形成法检测其活性明显优于文献报道的结果，体内对小鼠H22肝癌、Lewis肺癌、C26结肠癌有显著疗效。

发酵液
↓ 8000转/分，4°C，离心
上清液
↓ pH4.0，饱和硫酸铵，8°C
硫酸铵沉淀
↓ $H_2O$，8°C，透析
透析液
↓ 羟基磷灰石，pH6.8，8°C，洗脱
洗脱液
↓ 冷冻干燥
冻干品
↓ $H_2O$，Sephadex G-75，8°C，洗脱
洗脱液
↓ 冷冻干燥
力达霉素

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# 权 利 要 求 书

1. 抗肿瘤抗生素力达霉素的制备新方法，其特征是将力达霉素产生菌链霉菌 C-1027 在中性条件下振荡培养发酵，其活性物质经羟基磷灰石和 Sephadex G-75 分离、纯化得到成品。

2. 按照权利要求 1 所述的抗肿瘤抗生素力达霉素的制备新方法，其特征是力达霉素产生菌在高氏 1 号斜面培养基培养，28℃，7—10 天，取一小块接种一级种子（发酵培养基成分包含：淀粉，玉米浆，血胨，葡萄糖，无水硫酸镁，碘化钾，玉米面，碳酸钙等，pH7.0），28℃，旋转摇床培养 48 小时，再转种为二级种子，发酵培养基同一级种子，28℃，往返摇床培养 18 小时，上发酵罐，28℃，搅拌 400 转/分，pH6.5—7.0，发酵 96 小时，得到所需要的发酵液。

3. 按照权利要求 1 所述的抗肿瘤抗生素力达霉素的制备新方法，其特征是将力达霉素产生菌发酵液离心，上清液经硫酸铵沉淀，透析或超滤脱盐，羟基磷灰石柱吸附，洗脱除杂质，Sephadex G-75 柱层析分离，进一步洗脱除杂质，冷冻干燥得到抗肿瘤高活性力达霉素白色粉末精制品。

# 说　　明　　书

## 抗肿瘤抗生素力达霉素的制备新方法

本发明涉及一种大分子肽类抗肿瘤抗生素力达霉素的制备新方法。

力达霉素产生菌 C-1027 是从我国湖南省潜江县土壤中分离得到的链霉菌属菌株（中国医学科学 院医药生物技术研究所菌种保藏室提供），用精原细胞法为向导，微生物学方法追踪，从 2000 株放线菌的发酵液中发现，它不仅具有较强的抗瘤活性，还具有抗革蓝氏阳性菌及革蓝氏阴性菌的作用（C-1027 物质 1987 年申请了日本专利，特愿昭 62-160279，又于 1987 年申请了中国专利，申请号 88102750.6）。由该菌株分离得到的抗肿瘤活性物质力达霉素由一个蛋白肽和一个发色团以非共价键结合而成，二者可以拆分，发色团为其主要活性部位，但容易失活，蛋白肽对发色团活性具有保护作用。然而，现有的力达霉素制备技术比较复杂，工艺流程较长，用碱性离子交换树脂分离，力达霉素在分离过程中容易失活，且产量低，直接影响了该物质的抗肿瘤功效。

本发明之目的是，通过改进制备工艺,以获得稳定性好、纯度较高、疗效更佳的抗肿瘤抗生素力达霉素。

本发明之内容与要点：

一、菌种生物学特征鉴别

力达霉素产生菌 C-1027 菌株通常在链霉菌分类用的培养基中均能生长。

其形态特征是：孢子丝直至波曲（图 1），成熟的孢子丝链有 10—30 个或更多的孢子，孢子呈柱形，表面光滑（图 2）；

其培养特征是：在各种合成的或有机的培养基上，往往有浅粉软皮色乃至榛子色彩，基内菌丝一般无色，日久稍变乳脂色，无可溶性色素，亦不产生黑色素；

其生理特征是：产 $H_2S$、明胶液化、牛乳凝固及胨化、硝酸还原均为阳性反应，温度在 28—32℃ 时丰茂，生长迅速，45℃ 不生长；

其碳原利用情况：L—阿拉伯糖、D—木糖、D—葡萄糖、D—果糖、L—鼠李糖、D—甘露醇、D—半乳糖等均能促进生长良好；蔗糖、肌醇、乳糖、纤维素、卫茅醇、棉籽糖等对生长不起作用。

二、发酵培养

将力达霉素产生菌冷干管中加 0.7ml 无盐水，使之形成菌悬液，用白金耳接种于高氏 1 号斜面培养基培养，28℃，7—10 天，表面生长白色气生菌丝，取一

小块接种一级种子 100ml/500ml 三角瓶（发酵培养基成分可以为：淀粉 1%，玉米浆 0.5%，血胨 0.5%，葡萄糖 0.5%，无水硫酸镁 0.02%，碘化钾 0.06%，玉米面 1.5%，碳酸钙 0.4%，自来水配制，pH7.0，15 磅消毒），28℃，旋转摇床培养 48 小时，再转种 5%于 1000ml/5000ml 立瓶中，为二级种子，发酵培养基同一级种子，28℃，往返摇床培养 18 小时，上 200L 发酵罐，装量 100L，接种量 2%，加 0.03%泡敌为消沫剂，罐压 0.04，28℃，搅拌 400 转/分，气流 1/1，pH6.5－7.0，发酵 96 小时，得到所需要的发酵液。

发酵液生物学活性检测，八叠球菌为检定菌，采用杯碟法（单层培养基 10ml），抑菌圈直径 20－24mm，精原细胞法检测结果，X10000（发酵液稀释一万倍）仍为阳性。

三、分离提取

由上述发酵液产生的活性物质力达霉素，其分离提取新工艺主要采用羟基磷灰石柱层析，凝胶过滤层析方法进行。本发明采用羟基磷灰石柱层析，主要考虑到力达霉素是一个不稳定的化合物，对紫外光、热等敏感，在水溶液室温条件下其活性易丢失。由于力达霉素蛋白肽与发色团以非共价键结合，外界条件如有机溶媒、pH、离子强度和离子交换剂等影响它们的结合并引起发色团的失活。羟基磷灰石层析是通过多因素分离蛋白的：羟基磷灰石钙离子对蛋白质的亲和力、蛋白质的等电点、三维结构的差异、洗脱剂的离子特性、蛋白质极性基团和羟基磷灰石极性位点的相互作用等，这些综合因素使羟基磷灰石具有不同于离子交换层析的特殊分离效果而不影响力达霉素的活性。本发明同时简化操作流程，尽可能减少了活性物质力达霉素在水溶液中的滞留时间，避光操作，以免失活。

具体步骤是：将存在于发酵液中力达霉素物质离心去菌丝，取上清液加硫酸铵盐析，然后将含有力达霉素的沉淀物离心分离。将离心沉淀物溶于水或磷酸缓冲液，用透析或超滤脱盐，进一步用羟基磷灰石进行吸附，然后再洗脱除去杂质。洗脱液经冷冻干燥或超滤浓缩后，再经 Sephadex G-75 柱层析，进一步除去杂质，以获得高纯度的力达霉素。

其制备方法如工艺流程（图 3）所示。

鉴别：上述方法得到的力达霉素精制液冷冻干燥后，得到力达霉素的白色粉末。

本发明所得到的力达霉素为单一物质，SDS-聚丙烯凝胶电泳显示单一带（图 4），高压液相色谱为单一峰（图 5），此外，毛细管电泳色谱也为单一峰（图 6），均可得到证实。

本发明所得到的力达霉素物质，其理化性质与文献报道的力达霉素物质一致。

四、活性测定

精原细胞法测定，选用雄性昆明小鼠，睾丸内注射不同剂量的力达霉素，注射 3 天后处死动物，取标本，固定，包埋，切片，染色，最后在显微镜下观察。结果显示，力达霉素对精原细胞有强烈抑制作用，最低有效浓度为 0.001μg/ml，比文献报道的力达霉素（0.0039μg/ml）强 3.9 倍。

体外克隆生成测定法检测，取肿瘤细胞接种于 96 孔培养板中，每孔 50 个细胞，培养 24 小时后加不同浓度的力达霉素处理，7 天后倒置显微镜下数细胞集落数。结果显示，力达霉素对多种肿瘤细胞具有很强的杀伤作用，杀伤力在 $10^{-16}$mol/L 水平，包括人鼻咽癌 KB 细胞、人肝癌 BEL-7402 细胞、人结肠癌 HT-29 细胞和人胃癌 BGC-823 细胞等（见表一）。本发明制备的力达霉素比文献报道的力达霉素具有更强力的抗肿瘤活性，如对 KB 细胞，本发明的半数抑制浓度为 $2.6\times10^{-16}$ mol/L，文献报道的为 $1.0\times10^{-12}$ mol/L（0.0001μg/ml），对 HT-29 细胞，本发明的半数抑制浓度为 $1.1\times10^{-16}$ mol/L，文献报道的为 $1.3\times10^{-11}$ mol/L，比本发明均大 3 个数量级以上。

表一、力达霉素的体外抗肿瘤活性（克隆形成法测定）

| 肿瘤细胞系 | 半数抑制浓度（mol/L） |
|---|---|
| 人鼻咽癌 KB | $2.6\times10^{-16}$ |
| 人结肠癌 HT-29 | $1.1\times10^{-16}$ |
| 人肝癌 BEL-7402 | $3.2\times10^{-16}$ |
| 人胃癌 BGC-823 | $1.9\times10^{-16}$ |

体内小鼠移植性结肠癌的疗效实验，使用 BALB/c 小鼠，在腋窝皮下接种结肠癌 26 的瘤组织小块，接种 24 小时后静脉内注射不同剂量的力达霉素，1 次或 3 次，间隔 3 天，接种肿瘤 10 天后处死动物，取肿瘤块，称重，计算肿瘤抑制率。实验结果表明：力达霉素对小鼠结肠癌有非常明显的疗效，一次给药，0.15 mg/kg 剂量，肿瘤抑制率为 84%，三次给药，0.1 mg/kg 剂量，肿瘤抑制率为 94%。采用等毒性剂量（1/4 半数致死量 LD50 或 1/8 LD50），单次静脉内注射给药的治疗方案进行比较，在相当于 1/4 LD50 或 1/8 LD50 剂量时，力达霉素的肿瘤抑制率明显高于临床常用的抗肿瘤药物表阿霉素和丝裂霉素 C（见表二）。

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表二、力达霉素、表阿霉素和丝裂霉素 C 对小鼠结肠癌 C26 的影响

| | 剂　量<br>（mg/kg） | 相当毒性剂量<br>（剂量/LD50） | 肿瘤抑制率<br>（%） |
|---|---|---|---|
| 力达霉素 | 0.1 | 1/4 | 76 |
| | 0.05 | 1/8 | 70 |
| 表阿霉素 | 5.2 | 1/4 | 48 |
| | 2.6 | 1/8 | 38 |
| 丝裂霉素 C | 1.2 | 1/4 | 42 |
| | 0.6 | 1/8 | 39 |

药效学与急性毒性研究，结果表明：力达霉素对小鼠 H22 肝癌与 Lewis 肺癌均有高度疗效。静脉注射力达霉素的急性毒性 LD50 为 0.4mg/kg，力达霉素对 H22 肝癌的 ED50（50%抑瘤率）为 0.013mg/kg，化疗指数为 31；静脉注射丝裂霉素的 LD50 为 5mg/kg，丝裂霉素对 H22 肝癌的 ED50 为 0.78mg/kg，化疗指数为 6.4。力达霉素的化疗指数明显高于丝裂霉素。

本发明的优点与积极效果是：

制备流程短，比文献报道少四个步骤；产品稳定性好，纯度产率高，14.5mg/每升发酵液，比文献报道的 6.2mg/每升发酵液提高 1.3 倍；成本低，短流程和高产率减少了生产材料；产品活性强，比文献报道的力达霉素强 1000 倍以上（克隆生成半数抑制浓度），精原细胞法显示，力达霉素对精原细胞的抑制率比现有技术制备的强 3.9 倍。

力达霉素物质制备新方法实施例如下：

将力达霉素产生菌冷干管中加 0.7ml 无盐水，使之形成菌悬液，用白金耳接种于高氏 1 号斜面培养基培养，28℃，7—10 天，表面生长白色气生菌丝，取一小块接种一级种子 100ml/500ml 三角瓶（发酵培养基成分为：淀粉 1%，玉米浆 0.5%，血胨 0.5%，葡萄糖 0.5%，无水硫酸镁 0.02%，碘化钾 0.06%，玉米面 1.5%，碳酸钙 0.4%，自来水配制，pH7.0，15 磅消毒），28℃，旋转摇床培养 48 小时，再转种 5%于 1000ml/5000ml 立瓶中，为二级种子，发酵培养基同一级种子，28℃，往返摇床培养 18 小时，上 200L 发酵罐，装量 100L，接种量

2%，加 0.03%泡敌为消沫剂，罐压 0.04，28℃，搅拌 400 转/分，气流 1/1，pH6.5—7.0，发酵 96 小时，得到所需要的发酵液。

取发酵液 10 L，离心，取上清液，用 HCl 调 pH4.0，加硫酸铵 4.5 Kg，8℃搅拌 3 小时，析出的力达霉素离心分离（4℃，8000 转/分，15 分钟），沉淀物加 200 冷水溶解，透析，离心除去不溶物，上清液经羟基磷灰石柱吸附，0.001M 磷酸缓冲液（pH6.8）洗脱，活性部分冷冻干燥，得粗制品 1500 mg。粗制品溶于水，经 Sephadex G-75 柱层析，活性部分冷冻干燥，得到 145 mg 抗肿瘤高活性力达霉素白色粉末精制品，其理化性质及生物学特征如前所述。

附图说明：

图 1 是力达霉素产生菌 C-1027 菌株孢子丝（X400）；

图 2 是力达霉素产生菌 C-1027 菌株孢子形态（X1200）；

图 3 是力达霉素制备新工艺流程；

图 4 是 SDS-聚丙烯凝胶电泳谱；

其中：1—力达霉素

2—标准蛋白

图 5 是高压液相色谱；

图 6 是毛细管电泳色谱。

# 说 明 书 附 图

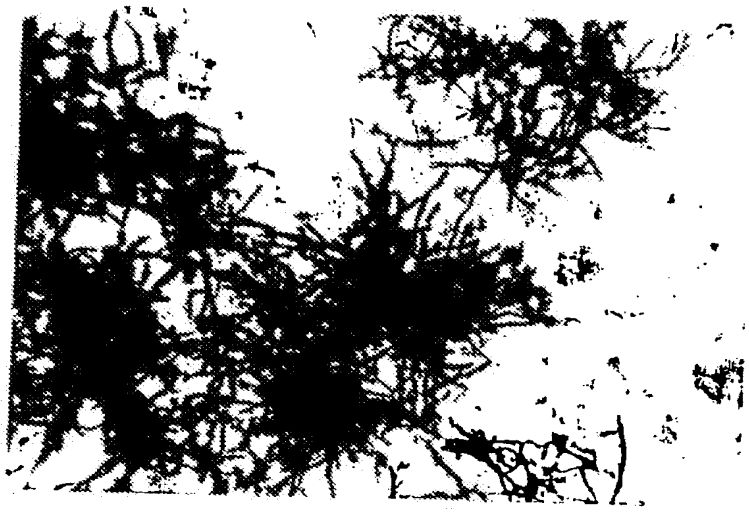

图 1

图 2

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发酵液

↓ 8000 转/每分钟，4°C，离心

上清液

↓ pH4.0，饱和硫酸铵，8°C

硫酸铵沉淀

↓ $H_2O$，8°C，透析

透析液

↓ 羟基磷灰石，pH6.8，8°C，洗脱

洗脱液

↓ 冷冻干燥

冻干品

↓ $H_2O$，Sephadex G-75，8°C，洗脱

洗脱液

↓ 冷冻干燥

力达霉素

图 3

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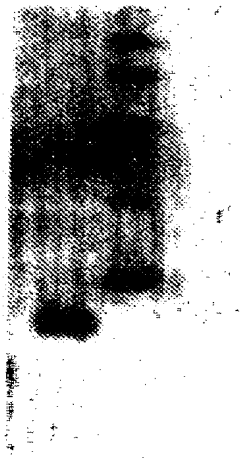



图 4

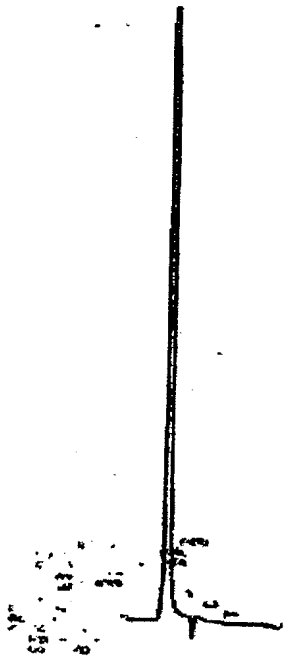


图 5

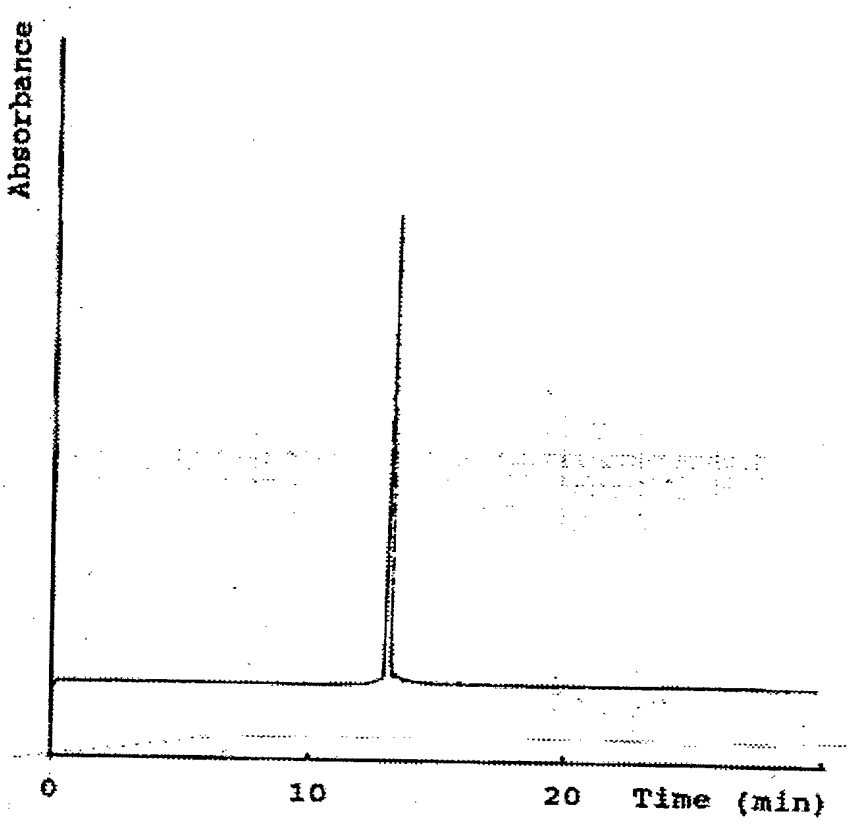
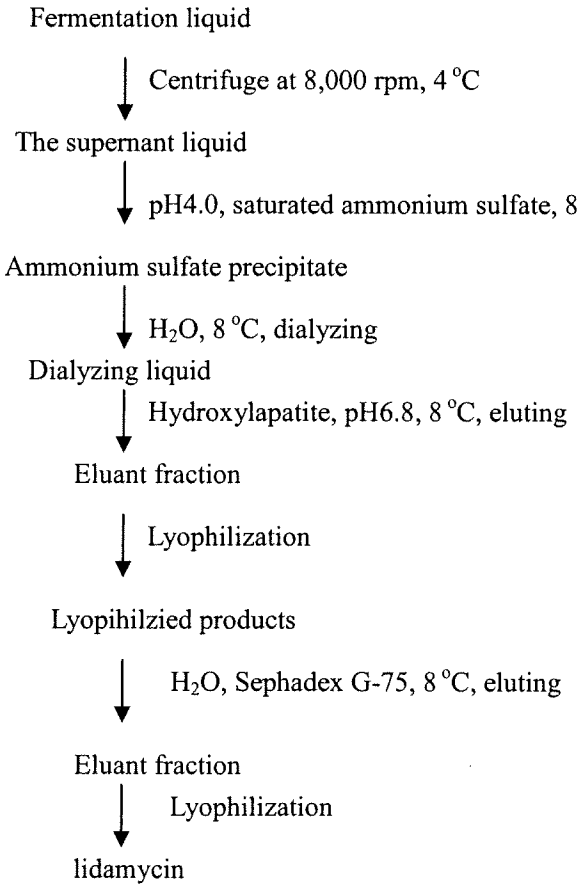



图 6

# A new method for the preparation of antitumor antibiotic lidamycin


## ABSTRACT

The present invention relates to a new method for producing a macromolecular antitumor antibiotic peptide lidamycin. Because of the non-covalent association of the peptide and chromophore in lidamycin molecule, external condition such as organic solvents, pH, eluent, iron charateristic, lights and heats, etc. can influence their association and arouse the inactivation of chromophore. By use of absorption chromatography on hydroxyapatite column in dark room, to short the flow process and operation, the special isolation effects were observed. Not only purity and stability but also the antitumor activity of the products were improved. The experiments of biological activity by spermatogonial assay and clonogenic assay demonstrated that the activity was higher than that of data reported in previous papers, and which showed prominent therapeutic effects to hepatoma 22, Lewis lung cancer, and colon carcinoma C26 in mice in vivo.


## CLAIMS

1. A new method for producing antitumor antibiotic lidamycin comprises the following main points: the lidamycin-producing *streptomyces globisporus* C-1027 was cultured and fermentated at neutral conditions, and the product was obtained after isolation and purification of the active substance by hydroxylapatite and Sephadex G-75 column.
2. The new method for producing antitumor antibiotic lidamycin of claim 1 comprises the

following steps: the lidamycin-producing strain was cultured on slant of Gause's No.1 medium at 28°C for 7-10 days; Then a small piece of medium above as the first-class seed was inoculated into the medium containing starch, corn syrup, blood peptone, glucose, $MgSO_4$, KI, corn meal, $CaCO_3$, etc, pH 7.0, at 28 °C , and cultured on a rotary shaker for 48 h; The obtaining second-class seed was transferred to the same medium and cultured on a shaker for 18 h at 28 °C, and the resulting fermentation liquid was obtained after culturing for 96 h and stirring at 400 rpm by use of fermentation tank.

3. The new method for producing antitumor antibiotic lidamycin of claim 1 comprises the following steps: the fermentation medium of lidamycin-producing strains was centrifuged and the supernatant was precipitated by adding ammonium sulfate; then after the ordinal steps of desalting by dialysis or ultrafiltration, absorption by hydroxylapatite column, eluting, chromatography by Sephadex G-75, eluting for removing impurities, and lyphilization, the refined lidamycin with potent antitumor activity was obtained as a white powder.

## SPECIFICATIONS

The present invention relates to a new producing method for antitumor antibiotic lidamycin. The producing strain C-1027 of lidamycin belonged to genus *Streptomyces* and was isolated from a soil sample collected in Qian-jiang county, Hu-nan province, China (The strain was preserved by Chinese Academy of Medical Science, Institute of Medicinal Biotechnology). The strain C-1027 was found from approximate 2,000 fermentation broths of actinomycete strains by use of spermatogonial assay and additionally by use of microbiogical tests. The fermentation broth of strain *Streptomyces globisporus* C-1027 was observed to have not noly activity of antitumor but also inhibition the growth of Gram-positive and Gram–negative bacteria (The patent of C-1027 was applied in Janpan in 1987, application No. 62-160279, and the corresponding Chinese patent was also applied in 1987, application No.88102750.6). The lidamycin with antitumor activity isolated from the strain consists of a non-covalent association of peptide and chromophore which can be separated. The chromophore is labile and is the main active moiety, and the peptide can preserve the activity of the chromophore. However, the existing procedure employed for the production of lidamycin was complex and time-comsuming by use of basic anion exchange resin for isolation. Lidamycin was prone to be deactivated and its yield was low in the process, which directly influenced the antitumor efficacy.

The aim of this invention is obtaining the antitumor antibiotic lidamycin with stability, high purity, and better therapeutic effects by improving the production method.

The main contents and points of the present invention are:

1. Biological properties of Strain

The producing strain C-1027 of lidamycin can grow in the usual culture medium used for classifying *Streptomyces*.

The properties in shape were described: the mycelium was straight to flexious appearance(figure 1), the mature spore chain generally had 10 to 30 or more than 30 spores per chain, and the spores were cylindrical with smooth surface(figure 2).

The culture characteristics were shown: pinkish-buff color even hazel color occurred on various synthetic or organic agar media; the substrate mycelium was usually colorless, became the color of cream in aged cultures, and no soluble pigment and melanin was produced.

The physiological characteristics were described: the $H_2S$ production, gelatin liquefaction, skim

milk coagulation, peptonization, and nitrate reduction were all positive. The strains grew abundantly and rapidly when the temperature was 28~32 °C, and no growth were observed at 45 °C.

The utilization of carbon sources was shown: L-arabinose, D-xylose, D-glucose, D-fructose, L-rhamnose, D-mannitol, and D-galactose can promote the growth of the strain. But for sucrose, inositol, lactose, cellulose, dulcitol, and raffinose, etc, there were no effects.

2. Fermentation

0.7 ml salt-free water was added into the lyophilized tube containing the lidamycin-producing strain, and the obtaining bacterial suspension was inoculated into the slant of Gause's No.1 medium with a platinum loop and cultured at 28°C for 7-10 days. Then a small piece of white aerial mycelium as the first-class seed was inoculated into 100 ml /500 ml culture flask. It could contain the following medium: 1% starch, 0.5% corn syrup, 0.5% blood peptone, 0.5% glucose, 0.02% $MgSO_4$, 0.06% KI, 1.5% corn meal, 0.4% $CaCO_3$ , preparied by adding main water, pH 7.0, sterilization at 15 pounds, and cultured for 48 h on a rotary shaker. The obtaining second-class seed was transferred into 1000 ml /5000 ml culture flask containing the same medium as the first-class one by a volume of 5%, and cultured at 28 °C for 18 on a shaker. Then the fermentation liquid was transferred by a volume of 2% into a 200 L fermentation tank containing 100 L of the same medium with 0.03% defoaming agent, and cultured for 96 h with a 0.04 tank pressure, 1:1 airflow, 400 rpm stirring speed, at pH 6.5-7.0. Then the resulting fermention liquid was obtained.

The biological activity of fermentation liquid was monitored by cylinder plate method and using sarcina as a test strain (single layer medium 10ml) . The diameter of anti-bacterial zone was 20-24 mm, and the results of spermatogonial assay showed that X10,000 (the fermentation was diluted 10000-fold ) were still positive.

3. Separation and extraction

Lidamycin was the active substance produced from the above-mentioned fermented broth. The new separation and extraction processes of lidamycin were performed mainly by hydroxylphosphorite column chromatography and gel filtration chromatography. The present invention adopted the hydroxyapatite column chromatography mostly considering the fact that lidamycin was a kind of unstable compound which was sensitive to ultraviolet light, heat and so on. Furthermore, the cromophone easily lost its activity in aqueous solution at room temperature. Because the protein peptide and chromophore of lidamycin were connected each other through non-covalent bond, so the exterior conditions such as organic menstruum, pH value, ionic strength, ion exchange agent, and so on , could affect their binding and trigger the inactivation of the chromophore. Hydroxyapatite column chromatography separated the proteins through multi-factors according to their affinity against the calcium ions in hydroxyapatite, the isoelectric points of the peptide, difference in three-dimensional structures, ionic characteristics of the eluent, the interaction between polar groups of proteins and their polar sites in hydroxyapatite, etc. All these integrated factors endowed the hydroxyapatite with specific separation efficacy different from that of ion exchange chromatography but didn't affect the activity of lidamycin. At the same time, the present invention also simplified the manipulation procedures and shortened the retention time of lidamycin in aqueous solution as well as possible. All of the manipulation should be carried out in the dark, which would protect lidamycin from the inactivation.

The detailed steps were illuminated. The fermented broth containing lidamycin was centrifuged

to remove the mycelium. To the supernatant, ammonium sulfate was added to precipitate lidamycin. Then, the precipitate consisting of lidamycin was collected by centrifugation and dissolved in water or phosphate buffer followed by dialysis or superfiltration to remove the salts. The resulting material was further adsorbed by hydroxyapatite and then eluted to remove the impurities. The eluate was lyophilized or concentrated by superfilter system and then applied onto Sephadex G-75 column to further wipe off the impurities in order to obtain the highly pure lidamycin.

The process flow of preparative methods was showed in Figure 3.

Accreditation: The white powder of lidamycin was obtained after the lyophilization of the refined solution using the above-mentioned methods.

Lidamycin obtained in present invention belonged to the simple component substance. Lidamycin gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as shown in Figure 4 and a single peak on high pressure liquid chromatography (HPLC) as revealed in Figure 5. In addition, capillary electrophoresis also showed a single peak in Figure 6. All of these data indicated the homogeneity of lidamycin.

For the physicochemical properties, lidamycin obtained in the present invention was accordance with lidamycin reported in the literature.

4. Determination of the Activity

Spermatogonial Assay

The male Kunming mice were injected intratesticularly with different dose of lidamycin for each spermatospore testes. Day 3 after injection, mice were sacrificed and the specimens of testes were fixed, embedded, sliced, stained and finally observed microscopically. Results showed that lidamycin exhibited an extremely inhibitory effect on the spermatospores with a minimal effective concentration of 0.001 μg/mL which was 3.9 fold stronger than that of lidamycin (0.0039 μg/mL) reported in the literature.

*In vitro* Clonogenic Assay

Tumor cells were seeded in 96-well plates with 50 cells per well. After incubation of 24 hours, various concentration of lidamycin was added into the well. Seven days after cell seeding, cell colonies were counted under an inverted microscope. Results indicated that lidamycin was extremely potent against cultured human tumor cells with the cytotoxic activity of $10^{-16}$ mol/L such as human nasopharyngeal cancer KB cells, human hepatoma BEL-7402 cells, human colon carcinoma HT-29 cells, human gastric cancer BGC-823 cells and so forth, as displayed in Table 1. The antitumor activity of lidamycin prepared in the present invention was much stronger than that of lidamycin reported in the literature. For KB cells, the concentration for 50% colony inhibition ($IC_{50}$) of lidamycin in this invention and the previous literature were $2.6\times10^{-16}$ mol/L and $1.0\times10^{-12}$ mol/L (0.0001 μg/mL), respectively. The corresponding $IC_{50}$ values were $1.1\times10^{-16}$ mol/L in this inwention and $1.3\times10^{-11}$ mol/L in the previous literature for HT-29 cells. The $IC_{50}$ values of lidamycin reported in the literature were 3 orders of magnitude more than those of lidamycin prepared in this invention.

**Table 1 *In vitro* antitumor activity of lidamycin (determined by clonogenic assay)**

| Human tumor cell lines | The concentration for 50% colony inhibition (mol/L) |
|---|---|
| Nasopharyngeal cancer KB cells | $2.6\times10^{-16}$ |
| Colon carcinoma HT-29 cells | $1.1\times10^{-16}$ |

| | |
|---|---|
| Hepatoma BEL-7402 cells | $3.2\times10^{-16}$ |
| Gastric cancer BGC-823 cells | $1.9\times10^{-16}$ |

*In vivo* Therapeutic Efficacy on the Transplantable Colon Carcinoma in Mice

A small piece of tumor tissue from murine colon carcinoma 26 was subcutaneously inoculated into the axilla of the BALB/c mice. After 24 h inoculation, different dose of lidamycin was administered intravenously once or three times with a 3-day interval. Ten days after tumor inoculation, the mice were executed and the tumor cakes were weighed. Further, the tumor inhibition rates were calculated. Experimental results indicated that lidamycin showed a remarkable therapeutic efficacy against murine colon carcinoma. The tumor inhibition rate was 84% for the single injection with the dose of 0.15 mg/kg, while the tumor inhibition rate was 94% for the three-time administration with the dose of 0.1 mg/kg. Next, the therapeutic protocols with the equal toxicity dose method (quarter of lethal dose $LD_{50}$ or eighth of lethal dose $LD_{50}$) were compared for single intravenous administration. As shown in Table 2, the tumor inhibition rates of lidamycin were evidently higher than those of epirubicin and mitomycin C that were the clinically frequently-used antitumor drugs when the injection doses of drugs were equivalent to the quarter or eighth of lethal dose $LD_{50}$.

**Table 2 Effect of lidamycin, epirubicin and mitomycin C on murine colon carcinoma 26**

| | Dose (mg/kg) | Equal toxicity dose (dose/$LD_{50}$) | Tumor inhibition rate (%) |
|---|---|---|---|
| Lidamycin | 0.1 | 1/4 | 76 |
| | 0.05 | 1/8 | 70 |
| Epirubicin | 5.2 | 1/4 | 48 |
| | 2.6 | 1/8 | 38 |
| Mitomycin C | 1.2 | 1/4 | 42 |
| | 0.6 | 1/8 | 39 |

Studies on the Pharmacodynamics and Acute Toxicity

Results indicated that lidamycin showed a highly therapeutic efficacy against murine hepatoma H22 and Lewis lung cancer. The $LD_{50}$ value of lidamycin injected intravenously was 0.4 mg/kg in the acute toxicity test. The $ED_{50}$ value (50% of tumor inhibition rate) of lidamycin against hepatoma H22 was 0.013 mg/kg and the chemotherapeutic index was 31. The $LD_{50}$ value of mitomycin injected intravenously was 5 mg/kg and its $ED_{50}$ value was 0.78 mg/kg against hepatoma H22 with the chemotherapeutic index of 6.4. So, the chemotherapeutic index of lidamycin was remarkably higher than that of mitomycin.

Advantages and Active Effect of the present invention

The preparative procedures offered in the invention patent were 4 steps shorter than those reported in the literature. The product was provided with better stability, higher purity and production rate. 14.5 mg of lidamycin could be achieved in one liter of fermented broth which was 1.3-fold higher than that was ever reported (6.2 mg/L) . It was the lower cost, shorter procedures and higher productivity that reduced the means of production. Additionally, the product also showed very strong activity. In terms of $IC_{50}$ values of cloning producing, the cytotoxicity of lidamycin was 1000-fold more potent than that of lidamycin ever reported in the literature.

Spermatogonial assay revealed that the inhibition rate of lidamycin to spermatospores was 3.9-fold stronger than that of lidamycin prepared using the existing techniques.

New Method of Lidamycin Preparation Implemented in the Present Invention.

0.7 mL of salt-free water was added into the freeze-drying tube containing the lidamycin-producing strain to form the suspending solution. The resulting solution was inoculated onto the slant of Gauze No.1 agar medium with a platinum loop and cultured at 28℃ for 7-10 days when white aerial mycelia grew good on the surface of medium. A small piece of medium containing spores and aerial mycelia was inoculated into 100 mL of the first-grade seed medium consisting of starch 1%, corn syrup 0.5%, blood peptone 0.5%, glucose 0.5%, $MgSO_4$ 0.02%, KI 0.06%, corn meal 1.5%, and $CaCO_3$ 0.4%, pH7.0, sterilization at 15 pounds in a 500-mL Erlenmeyer flask. The flask was incubated at 28 ℃ for 48 hours on a rotary shaker. Then, 5% of seed culture thus obtained were transferred to 1000 mL of the fermentation medium as the same as the seed medium in a 5000-mL Erlenmeyer flask. The 5000-mL flask was incubated at 28℃ for 18 hours on a reciprocating shaker to form the second-grade seed. The fermented broth needed was obtained through 96-hour fermentation in a 200-L fermentation tank with the cultured conditions composing of fermentation medium 100 liters, inoculation amount 2%, antifoam agent 0.03%, pressure 0.04 MPa, temperature 28℃, agitation rate 400 rpm, airflow 1/1 and pH 6.5-7.0.

10 liters of fermented broth were centrifuged. The supernatant was adjusted to pH 4.0 with HCl and 4.5 kg of ammonium sulfate were added. The mixture was stirred for 3 hours at 8℃. Precipitated lidamycin was separated by centrifugation at 8000 rpm, 4 °C, 15min. The precipitate was dissolved in 200-mL cold distilled water followed by dialysis and centrifugation to remove the indissoluble. The resulting supernatant was applied on the hydroxyapatite column. After the elution with phosphate buffer (0.001M, pH6.8), the active fractions were lyophilized and 1500 mg of crude material were obtained. Next, this crude material was dissolved in water and chromatographed on Sephadex G-75 column. 145 mg of white powder lidamycin with highly effective antitumor activity were obtained after the lyophilization of the active fractions. Results showed that its physicochemical and biological properties were the same as those depicted before.

**The Description of the Attached Figures**

Figure 1 The spore filaments of lidamycin-producing strain C-1027 (×400)

Figure 2 The spore morphology of lidamycin-producing strain C-1027 (×1200)

Figure 3 New process flow chart of lidamycin preparation

Figure 4 Analysis of lidamycin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis Lane 1, lidamycin; Lane 2, the standard proteins.

Figure 5 HPLC chromatograpm of lidamycin

Figure 6 Analysis of lidamycin by capillary electrophoresis

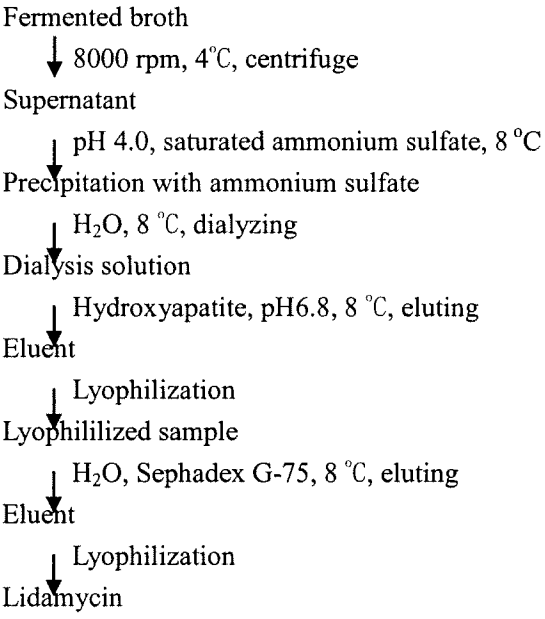

Fermented broth
8000 rpm, 4°C, centrifuge
Supernatant
pH 4.0, saturated ammonium sulfate, 8 °C
Precipitation with ammonium sulfate
$H_2O$, 8 °C, dialyzing
Dialysis solution
Hydroxyapatite, pH6.8, 8 °C, eluting
Eluent
Lyophilization
Lyophililized sample
$H_2O$, Sephadex G-75, 8 °C, eluting
Eluent
Lyophilization
Lidamycin

**Fig. 3**